

REMARKS

Reconsideration of the rejections set forth in the Office action mailed July 16, 2003 is respectfully requested.

I. Rejections under 35 U.S.C. §103(a)

Claims 28-48 (all pending claims) were rejected under 35 U.S.C. §103(a) as being unpatentable over Zalewski *et al.*, U.S. Patent No. 6,159,946, in view of Kobayashi *et al.* (*Osaka Daigaku Zasshi* 47(6-12), Abstract, 1995), Summerton *et al.* (U.S. Patent No. 5,378,841), Agrawal *et al.* (U.S. Patent No. 5, 912,332), and Burger (WO 98/46740). The rejections are respectfully traversed in light of the following remarks.

A. The Invention

The applicant's invention, as embodied in independent claim 28, is directed to a method of treating a vascular injury site in a patient by reducing restenosis at the site. The method comprises administering to the patient, by intravascular delivery directly to the vascular injury site, in an amount effective to reduce restenosis in the patient, a morpholino antisense compound having (i) from 8 to 40 nucleotides, including a targeting base sequence that is complementary to a region that spans the start codon of a human c-myc mRNA gene, and (ii) uncharged, phosphorus-containing intersubunit linkages.

Applicants emphasize that a "patient" is distinct from an animal model; see, for example, the specification at page 15, lines 10-11 ("administered to a patient **or** in an animal model"; emphasis added).

As discussed in the previous response, a recently completed Phase II clinical study, described in a Declaration by co-inventor Dr. Dwight Weller, showed efficacy in a patient population having existing recurrent restenosis following PTCA, and selected based on criteria targeting patients with a high probability of restenosis. As shown in the Declaration, patients receiving an anti-c-myc morpholino oligomer, as set forth in the claims, showed significantly less reocclusion than patients receiving a subtherapeutic dose or receiving no oligomer. No drug-related serious adverse effects were observed.

B. The Cited Art

Zalewski et al., cited previously, discloses the use of a phosphorothioate-linked oligonucleotide targeting c-myc mRNA in an *in vivo* porcine model of coronary angioplasty. The data showed that the oligonucleotide "significantly reduced neointimal formation" in this animal model (Example 11). The cited reference does not, however, disclose efficacy of the phosphorothioate oligonucleotide in a patient.

Kobayashi et al. discloses a phosphorothioate-linked oligonucleotide having a 27-nucleotide sequence which includes the 20-nucleotide sequence disclosed by the applicants as SEQ ID NO:1. The phosphorothioate oligonucleotide of Kobayashi was reported to suppress the tumor growth of gastric cancer cells and colon cancer cells transplanted in nude mice.

Summerton et al. discloses uncharged-backbone morpholino oligomers and their benefits over native RNA or DNA oligonucleotides as antisense agents.

Agrawal et al. is cited for its disclosure of a triethylene glycol solubility-enhancing group.

None of the foregoing three references, all of which were cited previously, provides any guidance as to suppression of restenosis in a patient.

Burger, cited for the first time, teaches the use of a morpholino oligomer having a nucleotide sequence directed to CMV (cytomegalovirus) for inhibition of restenosis.

The therapeutic approach described in Burger targets the DNA of an exogenous virus (CMV), which is completely different from the target of the present claims, an endogenous proto-oncogene (c-myc). Suppression of expression of these sequences would be expected to produce different physiological effects on a molecular level. Even though the targeted disease state is the same, the two treatment approaches are completely different. The teachings of Burger therefore would not provide any expectation of success in the use of an antisense oligomer targeting c-myc for suppression of restenosis in a patient, particularly in view of the overall state of the art, as discussed below.

C. The Weller Declaration

The above-referenced Weller Declaration, submitted on May 1, 2002, pointed out that a

clinical trial employing the phosphorothioate oligonucleotide disclosed in Zalewski *et al.* failed to show any efficacy in inhibiting post-PTCA restenosis in a patient population (as reported in Kutryk *et al.*, *J. Amer. Coll. Cardiology* **39**:281-7, 2002, enclosed with previous response).

Regarding this Declaration, the Examiner stated that "Applicants have not provided evidence that the conditions disclosed in Kutryk *et al.* were identical to those disclosed in the Zalewski *et al.* U.S. Patent No. 6,159,946..." and that "one of skill in the art would not accept on its face that Kutryk *et al.* provides evidence of non-enablement in regards to the teachings of Zalewski *et al.*" (pages 2-3 of Office Action).

With regard to the first statement, it was never the Applicant's aim to establish that "the conditions disclosed in Kutryk *et al.* were identical to those disclosed in the Zalewski *et al.*". Since the former study was carried out in a human patient population, and the latter was carried out in a porcine animal model, the conditions were clearly not identical. Kutryk *et al.* was cited by the Applicant, rather, to establish that the method demonstrated by Zalewski in U.S. Patent No. 6,159,946 in an animal model was unsuccessful in a human patient population. (See Declaration at page 3, line 1.)

D. Indications of Nonobviousness: Failure of other and long felt need

The CAFC held, in *In re Dow Chemical Co.*, 837 F.2d 469, 5 USPQ2d 1529 (Fed. Cir. 1988), that "Recognition of need, and difficulties encountered by those in the field, are classical indicia of unobviousness."

As stated in a preliminary report assessing the safety and pharmacokinetics of the Zalewski oligonucleotide (Roque *et al.*, *Antisense & Nucleic Acid Drug Dev.* **11**:99-106, 2001; copy enclosed with response of May 1, 2003), "Coronary restenosis remains a vexing clinical problem" (page 99, Introduction). The paper notes some of the associated difficulties, including the "more complex human atherosclerotic lesions" as compared to porcine coronary arteries in model studies (paragraph bridging pages 103-104).

The review article "Restenosis: a challenge for pharmacology" (H. Bult, *Trends in Pharmacological Sciences* **21**:274-279, July 2000; copy enclosed) states that "Finding effective therapies to combat restenosis has been difficult because of the incomplete

understanding of the biology of restenosis and the lack of suitable animal models" (first paragraph). Under "Further limitations of animal models" (page 274), it states that "The response to injury in healthy arteries might follow a very different course of events from the clinical reality in the abnormal human coronary arteries that exhibit complex intimal lesions".

Another review article, "Novel approaches for the prevention of restenosis" (L. Gruberg *et al.*, *Expert opinion on investigational drugs* 9(11):2555-78, Nov 2000; summary enclosed) states that "Despite intensive investigation in animal models and in clinical trials, most pharmacological agents have been found to be ineffective in preventing restenosis after percutaneous balloon angioplasty or stenting. Although studies frequently report success in the suppression of neointimal proliferation in animal models of balloon vascular injury, few of them have been successful in clinical trials."

Thus, the impression in the art, several months after the time of filing, was that coronary restenosis in the clinical setting was a long standing and intractable problem, and that success in an animal model did not necessary transfer to success in human patients at risk for restenosis.

Accordingly, the combination of Zalewski *et al.*, which shows the effect of a phosphorothioate-linked anti-c-myc oligonucleotide in an animal model of restenosis, and Burger *et al.*, which teaches the use of an anti-CMV morpholino oligomer, without supporting data, would not provide a reasonable expectation that the presently claimed oligomers would effectively inhibit restenosis at a vascular injury site in a patient, as demonstrated by the data presented in the enclosed Declaration.

In view of the foregoing, the applicants respectfully request the Examiner to withdraw the rejections under 35 U.S.C. §103(a).

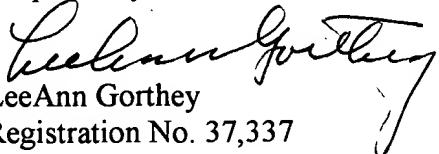
II. Conclusion

In view of the foregoing, the applicant submits that the claims now pending are now in condition for allowance. A Notice of Allowance is, therefore, respectfully requested.

If in the opinion of the Examiner a telephone conference would expedite the prosecution of the subject application, the Examiner is encouraged to call the undersigned at (650) 838-4403.

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Respectfully submitted,


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Restenosis: a challenge for pharmacology

Hide Bult

The quest for an anti-restenotic drug continues to be a major challenge in the field of cardiovascular pharmacology because most therapies with proven efficacy in experimental neointima models have failed to limit restenosis. Some drug classes, including glycoprotein IIb/IIIa antagonists, nitric oxide donors and the antioxidant probucol, have recently demonstrated potential benefits in clinical trials. Progress in the development of local delivery systems for administration of drugs, antisense oligonucleotides or genes, in combination with an improved understanding of the pathogenesis of restenosis holds promise for ultimate pharmacotherapy of this condition.

Since the late 1970s percutaneous transluminal coronary angioplasty (PTCA) has found widespread application in the therapy of coronary artery stenosis [i.e. narrowing of the lumen as a result of growth of an atherosclerotic plaque in the tunica intima (inner coat) of the vessel]. PTCA involves balloon dilatation of the obstructed segment to restore patency, thereby improving blood flow. Although the immediate success rate of PTCA has increased to more than 95%, long-term success remains limited by significant re-narrowing of the artery (restenosis) in 20–50% of patients within six months after the intervention. Finding effective therapies to combat restenosis has been difficult¹ because of the incomplete understanding of the biology of restenosis and the lack of suitable animal models.

Mechanisms of restenosis

Neointima

At first, research was focused on intimal thickening, often referred to as neointima formation. Commonly studied models involve gentle withdrawal of an inflated, low-pressure Fogarty balloon along normal rabbit or rat arteries to create injury of smooth muscle cells (SMCs) in the media. The SMCs start to proliferate (Box 1) and migrate to the intima, where cell division continues and matrix components are deposited. This healing process leads to formation of a neointima, which replaces the original intima consisting solely of a monolayer of endothelial cells.

Encouraged by promising results in neointima models, numerous clinical studies have examined whether systemic administration of a variety of pharmacological agents reduced the incidence of restenosis^{1,2}. The vast majority of these clinical trials could not demonstrate a reduction in the occurrence of restenosis in humans, whereas conflicting results were reported for fish oil, statins³ and glycoprotein (GP)IIb/IIIa antagonists (Table 1).

Other important mechanisms

It is now appreciated that acute elastic recoil, mural thrombosis and constrictive vascular remodelling might be equally important or dominant in the determination of the final lumen calibre^{4,5} (Box 1). The gentle denudation models lack the thrombotic and remodelling aspects, but these can be mimicked by repeated inflation of an oversized angioplasty balloon in animal arteries (Fig. 1). The significance of acute

elastic recoil and late constrictive remodelling is illustrated by 25–32% lower frequencies of angiographic restenosis in patients receiving coronary stents than in patients treated with conventional angioplasty¹. Intravascular ultrasound results indicate that the stent effectively opposes acute elastic recoil and late arterial shrinking. Although the stent elicits more pronounced neointima formation, the net result at six months remains better than with standard PTCA. Stent restenosis develops in 20–30% of the patients and might be more sensitive to therapies directed at inhibition of intimal thickening than restenosis following standard PTCA. However, trapidil, an antagonist of platelet-derived growth factor that reduced the frequency of restenosis in two small PTCA trials², failed to limit stent restenosis⁶. With 50% or more of the coronary interventions involving stents¹, prevention of neointima formation remains an important therapeutic target.

Further limitations of animal models

The response to injury in healthy arteries might follow a very different course of events from the clinical reality in the abnormal human coronary arteries that exhibit complex intimal lesions. SMC proliferation contributes significantly to neointima formation in animal models but in human restenotic coronary artery specimens proliferation occurs infrequently and at low levels⁷. Most of the volume of restenotic lesions appears to comprise extracellular matrix, and intimal expansion could occur largely by increased matrix deposition.

In addition, the importance of mediators involved in intimal thickening might differ among species. Angiotensin-converting enzyme (ACE) inhibitors and angiotensin II receptor antagonists suppress neointima in rat denudation models⁸, but they are ineffective in rabbit or porcine models of balloon angioplasty or stenting⁹. This suggests that the renin–angiotensin system does not play a pivotal role in the latter species.

Other differences concern the pharmacokinetic properties or the doses of the drug, which were sometimes very high in animal studies. Finally, strategies aimed at a single mediator with proven efficacy in simple denudation models are limited by the redundancy of cytokines, chemoattractants and mitogens in experimental and clinical PTCA (Ref. 8). Heterogeneity of receptor subtypes and of intracellular signalling systems confer further redundancy to the system.

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Box 1. Mechanisms of restenosis

Balloon angioplasty enlarges the vascular lumen by stretching less diseased sectors of the artery, fissuring of the plaque and separation of the plaque from the underlying tissue. Elastic recoil of the expanded artery causes a partial loss of the initial gain immediately after angioplasty (Fig. 1), and three delayed processes contribute to further lumen loss.

(1) Thrombus organization

The lacerations of the plaque or arterial wall promote (intra)mural thrombosis, which might result in peri-procedural myocardial infarction or death. The thrombus is subsequently organized by infiltrating phagocytes and α -smooth muscle cell (SMC) actin expressing cells. The latter proliferate and gradually replace the thrombotic material, while endothelial cells migrate and proliferate on the surface of thrombus and denuded media. However, the regenerated endothelium is unable to function normally, as indicated by inadequate vasomotion and formation of fresh mural thrombus even weeks after ballooning. Thrombus organization might contribute considerably to the bulk of the intima and late lumen loss in both animals^a and humans^{b,c}.

(2) Intimal thickening

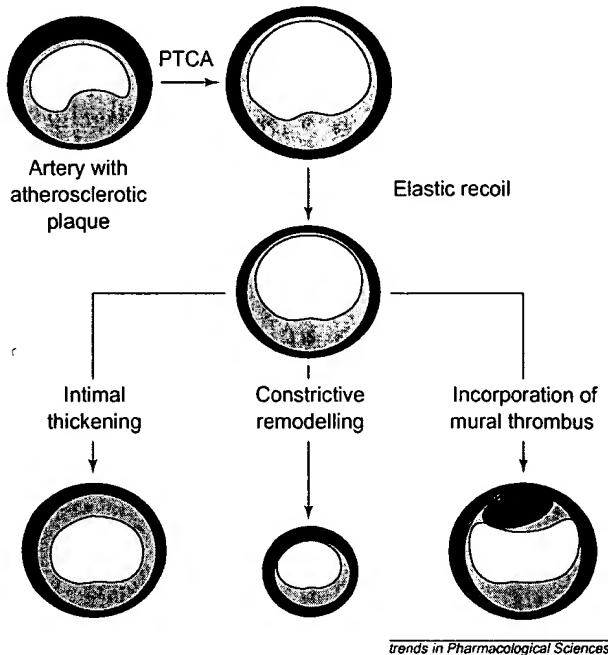
SMC injury evokes activation of a plethora of mitogens at the angioplasty site. Their receptors and second messenger systems are very heterogeneous, but they ultimately converge in a common pathway, during which the quiescent cells in the G0 phase enter the G1 (or interphase) of the cell cycle. The progression of the cell cycle is controlled by a variety of genes, such as *Fos*, *Myc*, *Myb*, *Rb*, etc. Suppression of any one of these genes will theoretically interrupt cell-cycle progression^{d,e}. When the balloon injury extends across the media, adventitial fibroblasts differentiate to myofibroblasts, which express α -SMC actin and synthesize collagen. These myofibroblasts might migrate through the arterial wall to become a major component of the neointima.

(3) Constrictive remodelling

Remodelling is defined as a change of the arterial size, judged from the area enclosed by the external elastic lamina. This could be a favourable enlargement compensating for the lumen loss as a result of intimal hyperplasia, or it might be unfavourable in terms of lumen preservation. In the latter case, the artery either cannot expand because of collagen-rich peri-adventitial scar tissue formed by adventitial myofibroblasts (see above) or even shrinks after injury. Experimental (see Fig. 1, main text) and clinical studies that evaluated both the structure of the vessel wall and the lumen diameter indicated that restenosis largely depends on chronic shrinkage of the vessel and not on intimal thickening.

Selected references

a Bosmans, J.M. *et al.* (1997) Fibrin(ogen) and von Willebrand factor deposition are associated with intimal thickening after balloon angioplasty of the rabbit carotid artery. *Arterioscler. Thromb. Vasc. Biol.* 17, 634–645



trends in Pharmacological Sciences

Fig. 1. Schematic view of the four mechanisms that lead to a loss of the lumen gain after a successful balloon angioplasty. Elastic recoil occurs immediately after percutaneous transluminal coronary angioplasty (PTCA), whereas mural thrombosis, intimal thickening and constructive remodelling contribute to delayed mechanisms underlying loss of luminal gain. The media is shown in blue, the intima is shown in mauve and the thrombus is shown in red.

b Bauters, C. *et al.* (1996) Mechanisms and prevention of restenosis: from experimental models to clinical practice. *Cardiovasc. Res.* 31, 835–846
 c Schwartz, R.S. (1998) Pathophysiology of restenosis: interaction of thrombosis, hyperplasia, and/or remodeling. *Am. J. Cardiol.* 81, 14E–17E
 d Pratt, R.E. and Dzau, V.J. (1996) Pharmacological strategies to prevent restenosis: lessons learned from blockade of the renin-angiotensin system. *Circulation* 93, 848–852
 e Topol, E.J. and Serruys, P.W. (1998) Frontiers in interventional cardiology. *Circulation* 98, 1802–1820
 f Scott, N. *et al.* (1996) Identification of a potential role for the adventitia in vascular lesion formation after balloon overstretch injury of porcine coronary arteries. *Circulation* 93, 2178–2187
 g Schwartz, R.S. *et al.* (1998) Artery size, neointima, and remodeling: time for some standards. *J. Am. Coll. Cardiol.* 32, 2087–2094

Encouraging systemic treatments

Two strategies to circumvent the complex interplay of multiple mediators are being explored with some success. One is aimed at pathways that are common to several agonists, whereas the other consists of employing broad-spectrum multipurpose weapons.

Glycoprotein IIb/IIIa as specific target

The final pathway to platelet aggregation common to all agonists is the formation of inter-platelet links through binding of fibrinogen to GPIIb/IIIa, an adhesion molecule on platelets. This discovery prompted studies of a chimerical

monoclonal antibody (abciximab) that blocked GPIIb/IIIa, and of competitive peptide and non-peptide inhibitors of GPIIb/IIIa. Clinical trials with these agents have consistently shown marked reductions in the risk of acute ischaemic events during the procedure. This early benefit was observed in all patients, irrespective of their risk profile or the interventional procedure (i.e. balloon angioplasty, stenting or atherectomy)¹⁰. Surprisingly, the inhibition of ischaemic events was maintained over long-term follow-up, which supports the idea that incorporation of early thrombus might contribute to the bulk of neointima^{11,12}. Although a reduced risk of late restenosis was not a consistent finding^{1,10},

Table 1. Pharmacological agents^a evaluated for their capacity to reduce the incidence of restenosis after balloon angioplasty

Class of pharmacological agent	Pharmacological agent	Refs
Anti-platelet agents	Acetylsalicylic acid, ticlopidine, thromboxane receptor antagonists, GPIIb/IIIa inhibitors ^c	1,2
Anticoagulants	Heparin, enoxaparin, warfarin	1
Phosphodiesterase type V inhibitor	Dipyridamole	1,2
Fish oil derivatives	n-3 fatty acids ^c	1
HMG-coA reductase inhibitors	Lovastatin, fluvastatin	1,3
Ca ²⁺ channel blockers	Nifedipine, verapamil, diltiazem	1
ACE inhibitor	Cilazapril	1,8
Immunosuppressive	Prednisolone	1
Antagonist of platelet-derived growth factor	Trapidil ^c	1,6
Analogue of somatostatin	Angiopeptin	1
Nitric oxide donor	Molsidomine ^b	20
Antioxidants	Probucol ^b , multivitamins	16

^aClinical data of angiotensin AT₁ receptor antagonists and endothelin receptor antagonists¹⁸ are not yet available.

^bBeneficial or ^cconflicting in randomized clinical trials.

Abbreviations: ACE, angiotensin-converting enzyme; GP, glycoprotein; HMG, 3-hydroxy-3-methylglutaryl.

results of two trials suggest that abciximab could reduce restenosis after balloon angioplasty¹³ or stenting¹⁴. Abciximab is not selective for GPIIb/IIIa and has equal affinity for the vitronectin receptor, an adhesion molecule involved in SMC adhesion, migration and proliferation. Furthermore, abciximab remains bound to platelets long after its rapid clearance from the plasma. Both factors could have contributed to the long-term beneficial effects of abciximab as opposed to other GPIIb/IIIa inhibitors¹. The potential of long-term administration of oral GPIIb/IIIa inhibitors to influence the frequency of restenosis has not yet been established but might be limited by a narrow therapeutic window with more dose-related bleeding in comparison to aspirin¹⁵.

Pleiotropic strategies: probucol and nitric oxide

At sites of tissue trauma resident SMCs and infiltrating leukocytes produce reactive oxygen species. These activate redox-sensitive transcription factors, such as nuclear factor κB, that trigger transcription of genes encoding cytokines, inducible NO synthase (iNOS) and other inflammatory proteins. Thus, interruption of oxidant-sensitive signalling systems could alleviate the inflammatory response invoked by balloon angioplasty. Indeed, treatment with the antioxidant probucol reduced the post-angioplasty restenosis incidence by ~40% and the lumen loss by ~70% compared with placebo¹⁶. However, administration of pharmacological doses of multivitamins (i.e. β-carotene, vitamin C and vitamin E) with antioxidant properties failed to limit restenosis. Therefore, it is uncertain whether the benefit of probucol is related to its antioxidant effects¹⁶. Moreover, the reduction of the levels of the beneficial high-density lipoprotein (HDL) particles by probucol raises the concern that probucol, although retarding restenosis of the target lesion, might adversely affect the atherosclerotic process at other sites.

NO also suppresses several restenotic processes. Its ability to promote adaptive arterial expansion¹⁷ and to inhibit SMC migration and proliferation, superoxide anion generation

and expression of adhesion molecules and cytokines might explain the beneficial effects of NO donors or L-arginine¹⁸ (Fig. 1) in animal models¹⁹. Treatment of patients with stable angina with the NO donor linsidomine (SIN-1) before balloon angioplasty followed by prolonged oral administration of molsidomine, the prodrug of SIN-1, led to a modest improvement in long-term lumen patency as a result of a better immediate result, without affecting clinical outcome or late restenosis²⁰. Thus, further exploration of the effects of NO donors on restenosis appears warranted.

Local therapy

PTCA creates a localized insult and the site is accessible during the procedure. Therefore, local treatment of the disease might yield maximal therapeutic efficacy with minimal systemic side-effects.

Local irradiation (brachytherapy)

A first approach is to bombard the vessel wall with β- or γ-irradiation immediately after angioplasty to disrupt the production of cell-cycle regulatory proteins (Box 1). With the correct irradiation dose the vascular SMCs remain viable but unable to replicate. Angioplasty studies in swine have demonstrated effective reductions of intimal proliferation using β- and γ-emitters, using catheter-based systems or stents to deliver the irradiation²¹. Studies in patients also indicate that catheter-based radiotherapy could lead to significant reductions of restenosis^{21–23}. A potential disadvantage is that the irradiation delays healing processes and restoration of an anti-thrombotic surface, thereby increasing the risk of late thrombotic events²⁴.

Coated stents

Much interest has been focused on designing stents coated with drug-eluting polymers. Studies in rabbit and swine have shown that the release kinetics were in accordance with the chemical characteristics of the selected compounds²⁵. However, the increased inflammatory response to the polymers is a potential drawback. Dexamethasone reduced the inflammatory response evoked by the polymer coating without influencing neointima formation. Yet, at four weeks the tissue concentration of dexamethasone still exceeded the plasma level 3000 fold, confirming the feasibility of slow drug release from polymer-coated stents. Stents eluting polyethylene glycol (PEG)-hirudin with prostacyclin, nitrosylated albumin, the tripeptide arginine glycine asparagine or methylprednisolone have been shown to suppress neointima formation²⁵.

Delivery catheters

Various catheter devices have been developed to enable local drug delivery or gene therapy. Unfortunately, experiments to rapidly instil agents into the vessel wall have met with low and variable efficiencies. Most of the infused material is distributed systemically and not localized to the angioplasty site or surrounding tissue. The development of reliable and highly efficient delivery catheters suitable for percutaneous instillation of agents into coronary arteries remains a crucial step to clinical application^{26,27}.

Antisense oligodeoxynucleotides

An approach to oppose cell-cycle progression consists of the local application of antisense oligodeoxynucleotides at the angioplasty site^{1,28}. These short DNA sequences are complementary to specific regions of mRNA. On binding to its target, the antisense DNA suppresses translation by causing steric hindrance of the interaction of ribosomes with mRNA. In addition, the DNA–RNA hybrid is more susceptible to degradation by RNase than single-stranded mRNA, which results in an increased clearance of target mRNA from the cell. In rats and swine, neointima development was reduced by injecting antisense oligonucleotides directed towards the mRNA encoding the short-lived cell-cycle proteins, such as MYB, CDC2 serine/threonine kinase, cyclin-dependent kinase 2 (CDK2) and proliferating cell nuclear antigen (PCNA)^{1,28}. A human trial to use an antisense targeted against the mRNA for myc is in progress¹.

Local gene transfer

Another strategy is based on gene transfer of cell-cycle specific inhibitory proteins directly into the proliferating SMCs (Ref. 29). To date, adenoviral vectors are by far the most efficient vectors to perform gene delivery, but substantial technical issues remain to be addressed before they can be applied to clinical restenosis. The first-generation recombinant adenoviruses evoke immune responses leading to inflammation, thereby increasing intimal hyperplasia. Furthermore, the high prevalence of pre-existing immunity to adenovirus constitutes another problem. Partly because of clearance of the transfected cells by the host immune system^{26,27,29}, the expression of the transfected genes is often transient; however, this transient expression might be sufficient to suppress restenosis.

Indeed, suppression of neointima formation has been achieved in animal models following adenoviral-mediated transfer of various cytostatic genes (e.g. genes encoding proteins p21, p53 and a dominant-negative form of H-Ras) to prevent cell-cycle entry. Cytotoxic approaches, in which the locally transfected gene converts a systemically administered prodrug to a potent inhibitor of DNA or RNA synthesis, leading to death of cells that have entered the cell cycle and paracrine 'bystander killing' of neighbouring cells, have also been successful in animal models^{26,27,29}.

The clinical effectiveness of anti-proliferative gene therapies and their advantage over local irradiation or local application of antineoplastic compounds, such as paclitaxel^{30,31} or mithramycin³², remain to be determined. Moreover, the focus on cell division might be inappropriate because proliferation might not be the primary pathological process in human restenosis^{7,27}.

Other targets of gene therapy

Migration of SMCs first requires activation of matrix metalloproteinases to dissolve the matrix cages and to enable locomotion. Local overexpression of the tissue inhibitor of matrix metalloproteinase-1 reduced neointima formation in the rat carotid artery model²⁹. Strategies that might limit thrombosis and reduce intimal thickening are the transfection of the thrombin inhibitor hirudin³³, or of cyclooxygenase 1 (Ref. 34) or prostacyclin synthase^{35,36} to augment prostacyclin biosynthesis.

Transfer of genes encoding NOS

NO exerts combined effects on intimal hyperplasia, platelet activation and constrictive remodelling (see above). Local overexpression of endothelial NOS (eNOS) has been found to inhibit intimal hyperplasia in response to denudation of the rat carotid artery^{37,38} or balloon angioplasty of swine coronary arteries³⁹. In the latter model there was a combined effect on vessel remodelling, leading to significantly larger lumen size. Similarly, seeding of rat smooth muscle transfected with the human gene encoding eNOS onto the surface of the denuded carotid artery led to neointima reduction and marked arterial dilatation⁴⁰. Gene transfer of iNOS has also been reported to suppress injury-induced myointimal hyperplasia of isolated porcine arteries *in vitro*, but NO biosynthesis and the anti-proliferative effect required the exogenous supply of tetrahydrobiopterin⁴¹. Several effects of NO are mediated by cGMP and local expression of C-type natriuretic peptide to stimulate the particulate guanylate cyclase markedly suppressed neointima formation in a denudation model⁴².

Endothelial regrowth

Endothelial cells produce other factors in addition to NO that could suppress restenosis. Local delivery of vascular endothelial growth factor (VEGF), a mitogenic and angiogenic factor, accelerated re-endothelialization and decreased intimal proliferation in rats. Similar results were obtained after local delivery of naked plasmid DNA encoding the

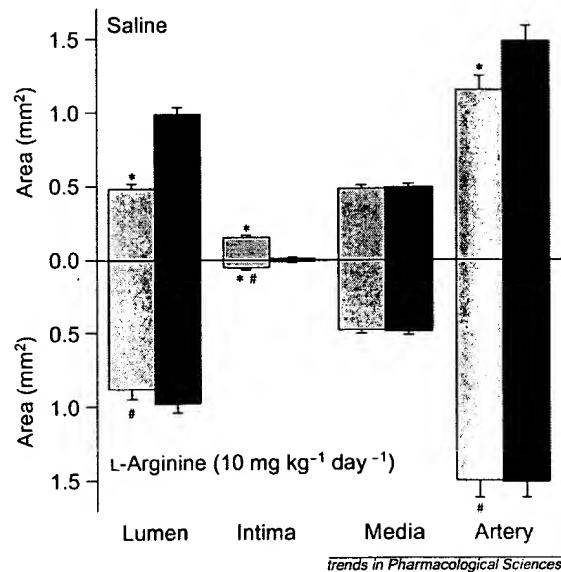


Fig. 1. The reduced lumen size of the rabbit carotid artery two weeks after repeated inflation of an angioplasty balloon was caused by arterial shrinkage and to a lesser extent by neointima formation (top panel) (light grey bars denote balloon angioplasty and black bars denote contralateral artery). After angioplasty, inducible nitric oxide synthase (iNOS) was expressed by macrophages invading the adventitia and mural thrombi, and continuous local perivascular delivery of the substrate L-arginine for two weeks suppressed both intimal thickening and constrictive remodelling in comparison to saline infusion, thereby increasing lumen diameter (bottom panel). **P* < 0.05, dilated artery different from contralateral control; #*P* < 0.05, L-arginine different from saline. Adapted, with permission, from Ref. 18.

165-amino-acid isoform of VEGF during balloon denudation⁴³ or stenting⁴⁴ of rabbit arteries. The accelerated endothelialization led to reduced thrombogenicity, improved endothelium-dependent relaxation and less intimal thickening. However, the experiment demonstrated prominent systemic vector delivery, because similar benefits were observed in the balloon-denuded contralateral artery, not subjected to gene transfer⁴³. Extravascular plasmid/liposome-mediated VEGF gene transfer around the rabbit carotid artery also reduced intimal thickening compared with β -galactosidase-transfected control arteries. The attenuation of intimal growth proceeded via a mechanism that involved VEGF-induced NO production from the endothelium⁴⁵. A clinical trial to test VEGF in peripheral artery angioplasty is in progress⁴⁶. Possible disadvantages of VEGF include stimulation of mitotic activity in SMCs. This could have contributed to the exacerbated neointimal proliferation in the canine denuded iliofemoral artery in response to left atrial administration of VEGF to stimulate coronary collateral development⁴⁷. Furthermore, by promoting neovascularization of the atherosclerotic plaque, VEGF could accelerate lesion growth or increase the risk of plaque rupture²⁹.

Concluding remarks

It is now appreciated that vascular shrinkage is a major determinant of the final lumen size after PTCA. In combination with ineffective dose levels, this might explain why drugs, which inhibit neointima in animal models, failed to be active against restenosis in the clinic. Although constrictive remodelling can be opposed by stent placement, in-stent restenosis as a result of intimal hyperplasia remains an important clinical problem. Basic research has led to a better understanding of potential drug targets and progress has been made in the design of local delivery systems and drug-eluting stents. Animal studies indicate that these techniques in conjunction with anti-neoplastic drugs, antisense oligonucleotides or gene transfer can reduce neointima formation by local control of cell proliferation, migration or matrix production. Although the efficacy of these approaches and their advantages or disadvantages in comparison to local irradiation remain to be determined in human restenosis, it gives hope that a combined mechanical and pharmacological therapy might ultimately limit restenosis.

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BOOK REVIEW

Comments and controversies about ENaC

Amiloride-sensitive Sodium Channels: Physiology and Functional Diversity

edited by Dale J. Benos, Academic Press, 1999. \$99.95 (xxiv + 384 pages)
ISBN 0 12 089030 5

This book is Volume 47 in the series *Current Topics in Membranes*. Written by knowledgeable investigators in the field, each chapter represents a thorough review of a focused topic through early 1999. This area of research is moving so quickly that several of the questions identified by the authors as being of major importance have already been answered or, in some cases, reframed since the chapter was written. Nevertheless, the book provides a valuable collection of the relevant research and current understanding of amiloride-sensitive Na^+ channels. The major areas that are covered include: (1) structure and function; (2) regulation; (3) Na^+ channels in the lung; (4) Na^+ channels involved in taste and sensation; and (5) clinical disorders (e.g. hypertension and cystic fibrosis).

Amiloride-sensitive Na^+ channels are not a homogeneous group. Although the major group of channels classified in this way are of the epithelial Na^+ channel (ENaC)/degenerin (DEG) family, a significant number of channels are included in the book simply because they are inhibited by amiloride. This inclusion, under the pharmacological umbrella of amiloride sensitivity, sometimes results in strange bedfellows. For example, there is a chapter devoted to Apx, a putative channel, which, when its mRNA is injected into oocytes, does not produce any channel activity. The author makes a valiant case for this protein being a channel, but the evidence is circumstantial at best.

Another group of channels, which is included for the sake of completeness,

is the nonselective cation channels. Although these channels are known to be important in sight and smell, and are regulated by cGMP, a major focus of the discussion is their characterization in the lung where their function is unclear. This group of channels is expressed in many tissues and undoubtedly plays a role in cell function. However, their precise role (even in the lung) is not clear, as this chapter demonstrates.

Perhaps the most important contribution of this book is the articulation of the controversies that surround several aspects of this general topic. One such controversy, discussed thoroughly in Chapters 2 and 3, relates to the stoichiometry of the ENaC complex. For this complex to function as it normally does in the colon and collecting duct, it requires three subunits (α , β and γ). The number of each of these subunits required for its 'normal' function is either three subunits of each type or two α -subunits and one each of the other two. The investigators who have addressed this question have done so thoroughly and carefully and have arrived at different conclusions. This 'controversy' has not been resolved since this text was published.

Novel approaches for the prevention of restenosis.

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Restenosis, the re-narrowing of the lumen of the coronary artery, in the months following a successful percutaneous balloon angioplasty or stenting, remains the main limitation to percutaneous coronary revascularisation. Serial intravascular ultrasound studies have shown that restenosis after conventional balloon angioplasty represents a complex interplay between elastic recoil, smooth muscle proliferation and vascular remodelling, while restenosis after stent deployment is due almost entirely to smooth muscle hyperplasia and matrix proliferation. Despite intensive investigation in animal models and in clinical trials, most pharmacological agents have been found to be ineffective in preventing restenosis after percutaneous balloon angioplasty or stenting. Although studies frequently report success in the suppression of neointimal proliferation in animal models of balloon vascular injury, few of them have been successful in clinical trials. Lately, the advent of endovascular radiation, new antiproliferative agents, recombinant DNA, growth factor regulators and novel local drug delivery systems have shown promising results. In the past five years, intracoronary radiation with gamma- and beta-emitting sources has been evaluated intensively with very encouraging results. This is the first potent non-pharmacological approach that has been successful in a large number of patients in controlling excessive tissue proliferation. It is very likely that a combination of stents and pharmacological and/or non-pharmacological inhibition of neointimal hyperplasia will likely result in further reductions in the incidence of restenosis. The continued attractiveness of percutaneous coronary revascularisation, as an alternative to medical treatment or bypass surgery for patients with coronary artery disease, will depend upon our ability to control the restenotic process. Due to the vast literature on the subject, this review will focus mainly on clinical trials that show the most promise and will highlight those that warrant further investigation. (160 Refs.)